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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Estrogens are well known to play an important role in both the onset and malignant progression of breast cancer. The content of estrogen receptors in breast tumors is a valuable predictor of whether a patient will respond to therapy with antiestrogens, such as tamoxifen and fulvestrant (ICI 182,780). Expression and activity of ER can be lost or impaired in antiestrogen-resistant breast cancer. The proposed studies are designed to test the overall hypothesis that the ubiquitin-like NEDD8 protein modification pathway represses estrogen action by facilitating degradation of ER protein. Perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of this pathway may prove to be a valid target for novel therapeutics. This study on mechanisms that regulate ER levels and activity are highly relevant to the development and progression breast cancer, including tumor progression to states of hormone independence and antiestrogen resistance. Receptor coregulators, such as Uba3, may represent a crucial point of control of estrogen action. Thus, understanding how coregulators influence the estrogen receptor is an area of research critical to understanding the tissue selective pharmacology of estrogens, tamoxifen and other SERMS and of the utmost relevance to therapies that target ER and other nuclear receptors.

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Fan M, Bigsby RM, Nephew KP 2003 The NEDD8 pathway is required for proteasome mediated degradation of human estrogen receptor-α and essential for the antiproliferation activity of ICI 182,780 in ER-positive breast cancer cells Mol Endocrinol 17:356-365 (*manuscript selected for the cover*).

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Copies of Abstracts.

1) Fan M, Long X, Bailey JA, Reed CA, Gize EA, Osborne E, Kirk EA, Bigsby RM, Nephew KP The activating enzyme of NEDD8 inhibits steroid receptor function. Keystone Symposium on Nuclear Receptor Superfamily, April, 2002
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INTRODUCTION: (Briefly, one paragraph, describe the subject, purpose and scope of the research)

Estrogen regulates diverse biological processes through estrogen receptors (ERα and ERβ) (1). Receptor levels and dynamics have a profound influence on target tissue responsiveness and sensitivity to estrogen, and receptor turnover rates provide estrogen target cells with the capacity for rapid regulation of receptor levels and thus dynamic hormone responses (2). In advanced stage breast cancers, estrogen receptor expression and activity can be lost or impaired, and the tumors are often resistant to endocrine therapies such as the the steroidal antiestrogens, ICI 182,780 and ICI 164,384 (3, 4).
We recently identified the NEDD8 activating enzyme, Uba3 as an ER-interacting protein and inhibitor of transactivation by steroid nuclear receptors (5). We further demonstrated that an intact neddylation pathway is required for Uba3-mediated inhibition of ER transcriptional activity (5). Taken together with recent reports linking the ubiquitin and NEDD8 pathways (6), our findings raise the intriguing possibility for a role of neddylation in ERα ubiquitination and degradation and suggest that disruptions in the NEDD8 pathway may contribute to the development of antiestrogen-resistance in human breast cancer. This proposal will test the overall hypothesis that the ubiquitin-like NEDD8 protein modification pathway represses estrogen action by facilitating degradation of ER protein. Perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of this pathway may prove to be a valid target for novel therapeutics.

BODY (describe the research accomplishments associated with each task outlined in the approved Statement Of Work)

The first task of the project was to determine the molecular mechanisms of ERα corepression by the NEDD8 pathway. Toward this goal, we have constructed Uba3 deletion mutants using PCR-mediated cloning techniques. These mutants lack one or both of the presumptive nuclear receptor interacting motifs (the so called NR boxes). The mutants have been cloned into the pGEX vector. The next step is to commence protein-protein interaction studies by performing GST-pulldown assays and examine the ability of full lengthUba3 and the Uba3 mutants to interact with ERα. Work toward this task is in progress.

Task 2 was to determine if ERα and ERα function is modified by APP-BP1 and Ubc12 and an NEDD8 target protein. First, we took a direct approach and determine if ERα is an NEDD8 target protein. We performed perform co-transfection experiments, co-immunoprecipitation assays and Western blot analysis and looked for NEDD8-ER complexes. We included various other components of the NEDD8 pathway, including co-transfecting Uba3, APPBP1, Ubc12 and various Cullin family members. We were never able to detect neddylated receptor (data not shown); therefore, we conclude that ER is not a direct substrate for modification by NEDD8.

Next, we tested the hypothesis that the neddylation pathway may act to restrict ERα activity by indirectly modulating receptor degradation. We transfected HeLa cells with ERα, alone or in combination with an expression vector for Uba3, APP-BP1, or Ubc12, or with an empty vector (pcDNA3); a green fluorescence protein (GFP) expression vector was cotransfected to serve as a means of normalizing transfection efficiency and sample preparations.

Steady-state levels of ERα protein were determined by Western blot analysis. Coexpression of Uba3 decreased ERα protein level but had no effect on GFP expression (Fig. 1A in manuscript in appendix). Treatment of the transfected HeLa cells with MG132, a specific proteasome inhibitor, blocked Uba3-stimulated down-regulation of ERα (Fig. 1B in manuscript in appendix), confirming that the Uba3-induced ERα degradation is through the 26S proteasome. Overexpression of APP-BP1 or Ubc12 had no significant effect on ERα protein levels (data not shown), a result consistent with our previous observation that Uba3 is the limiting factor in neddylation-associated inhibition of ERα transcriptional activity (25).

Next we tested the hypothesis that the neddylation pathway is required for ligand-mediated degradation of ERα. Estradiol stimulates ERα degradation through the ubiquitin-proteasome pathway (7-11), and having established a role for Uba3 in this process, it was important to assess whether neddylation pathway is required for ligand-induced degradation of ERα. To address this issue, we used a dominant negative mutant of Ubc12 (Ubc12C111S). Due to a single Cys-to-Ser substitution at the active Cys residue, Ubc12C111S forms a stable complex with NEDD8, resulting in sequestration of NEDD8 and inhibition of subsequent NEDD8 conjugation (12, 13). Dominant negative inhibition of NEDD8 conjugation by Ubc12C111S has been shown to impair efficient ubiquitination and protein degradation (14-17). Treatment of ERα transfected HeLa cells with estradiol resulted in a time-dependent decrease in ERα protein levels; receptor levels were reduced by 80% at 6-8 h. (Fig. 2A). In contrast, the effects of estradiol on receptor levels were less dramatic in cells expressing Ubc12C111S, producing a reduction of only 40% by 6-8 h (Fig. 2A in manuscript in appendix). Consistent with this observation, Uba3C216S, a dominant negative mutant of Uba3 (12, 13), also inhibited estradiol-induced ERα down regulation (Fig. 2B). Addition of the proteasome inhibitor MG132 prior to estradiol treatment completely abolished ligand-induced down-regulation of ERα (Fig. 2B in manuscript in appendix), confirming that exogenous ERα in HeLa cells undergoes proteasome-dependent degradation in response to estradiol. Collectively, these results demonstrate that a functional NEDD8 pathway is required for efficient, ligand-induced, proteasome-mediated degradation of ERα.

Having established a role for Uba3 and Ubc12 in ERα down-regulation, it was important to examine the effect of NEDD8 on receptor ubiquitination. HeLa cells were cotransfected with ERα and hemagglutinin (HA)-tagged ubiquitin, along with wild type Ubc12 or Uba3 or the corresponding mutant forms of these neddylation enzymes (Ubc12C111S or Uba3C216S). At 24 h posttransfection, cells were treated with MG132 or vehicle, followed by estradiol treatment. Immunoprecipitation assays using an anti-ERα antibody were performed and the levels of ubiquitinated ERα in the precipitated immunocomplex were assessed by Western blotting with an anti-HA antibody. The polyubiquitinated ERα exhibited a ladder of higher molecular weight species on the blot membrane (Fig. 3 in manuscript in appendix). Expression of dominant negative Ubc12C111S or Uba3C216S markedly decreased ERα ubiquitination in either the absence (Fig. 3, left panel) or presence of estradiol and MG132 (Fig. 3, right panel in manuscript in appendix), compared to cells transfected with control vector or wild type Ubc12 or Uba3. These results suggest that a functional neddylation pathway is required for the efficient ubiquitination of ERα.

Task 3 was to determine the effect of the NEDD8 pathway on breast cancer cell proliferation. To address this task, we generated a breast cancer cell line stably expressing a dominant negative Ubc12. The MCF7 human breast cancer cells express high levels of ERα and proliferate in response to estrogen treatment (18, 19), providing a model to study endogenous ERα function. To further investigate the role of neddylation in ERα function under physiological relevant conditions, we transfected Ubc12C111S into MCF7 cells and established the stable cell line MCF7/C111S. As a control, MCF7/Vec (MCF7 cells stably transfected with empty vector) was also established. Expression of the Ubc12C11S mutant protein in MCF7/C111S cells was confirmed by Western blotting and, consistent with a previous report (31), the mutant was detected as 26 and 31 kDa proteins (Fig. 4, lanes 3-8 in manuscript in appendix). In the regular growth medium containing phenol red and 10% fetal bovine serum (FBS), the level of ERα in MCF7/Vec cells was very low; after 3 days of culture in hormone-free medium containing 3% dextran-coated charcoal-stripped FBS (csFBS) and no phenol red, ERα expression was dramatically increased (Fig. 4, lanes 1 and 2 in manuscript in appendix). The culture medium (regular growth medium *vs* hormone-free medium) showed no effect on the expression level of Ubc12C111S. In three MCF7/C111S clones, receptor levels varied among the clones and, when cultured in growth medium, detectable ERα was seen in two of the three clones (Fig. 4, lanes 5 and 7 in manuscript in appendix). When cultured in estrogen-free medium, however, ERα levels were high in all three clones (Fig. 4, lanes 4, 6, 8 in manuscript in appendix).

In contrast to estradiol, which down-regulates ERα in target tissues through both transcriptional and posttranslational mechanism (20, 21), the pure antiestrogen ICI 182,780 causes ERα protein degradation without affecting ERα mRNA levels (3, 21). Based on our observations that the NEDD8 pathway is essential for ERα degradation in transfected HeLa cells (Fig. 2 in manuscript in appendix), it was of interest to examine the effect of the antiestrogen on ERα degradation in MCF7/C111S cells. Cells were cultured in hormone-free medium for 3 days prior to ICI 182,780 treatment. Under this condition, comparable amounts of ERα were observed in MCF7/C111S and MCF7/Vec cells (compare 0 h lanes in Fig. 5A in manuscript in appendix). Treatment with ICI 182,780 rapidly (by 1 h) decreased ERα levels in the MCF7/Vec cells; by 4 h post treatment, the levels of ERα were reduced by 95% (Fig. 5A in manuscript in appendix). In the MCF7/C111S cells, the effects of ICI 182,780 on ERα levels were much less dramatic (Fig. 5A in manuscript in appendix). Thus, although ER degradation was not completely inhibited by expression of the dominant negative Ubc12C111S, these results confirm our observations using transient transfection in Hela cells and further suggest that the NEDD8 pathway is required for efficient degradation of endogenous ERα. To examine the effect of another antiestrogen on ERα degradation in this system, cells were cultured in the presence of various doses of 4-hydroxytamoxifen (4-OHT) and ERα levels were examined. In both MCF7/Vec and MCF7/C111S cells, ERα levels remained unchanged or were slightly increased after treatment with 4-OHT (Fig. 5B in manuscript in appendix). Stabilization of ERα by tamoxifen has been reported by others(11), perhaps due to inhibition of the basal rate of ER degradation by the antiestrogen.

We next sought to disrupt the NEDD8 pathway and examine the response to antiestrogens in these breast cancer cells. Estradiol is mitogenic in MCF7 cells and stimulates

cell proliferation through activation of ERα (22). The pure antiestrogen ICI 182,780, on the other hand, blocks ERα-mediated transactivation and induces ERα protein degradation, resulting in growth inhibition of breast cancer cells (23). Because expression of Ubc12C111S inhibited ICI 182,780-induced ERα down-regulation (Fig. 5A in manuscript in appendix), we examined the growth inhibitory effect of ICI 182,780 in MCF/C111S cells. No significant difference was observed in basal cell proliferation rates between MCF7/C111S and MCF7/Vec cells in hormone-free medium (data not shown). Treatment with the antiestrogen (1 nM) inhibited the basal cell growth of MCF7 and MCF7/Vec cells (Fig. 6A in manuscript in appendix). In contrast, MCF7/C111S cells were partially resistant to ICI 182,780. Specifically, over an 8-day period, the antiestrogen inhibited the growth of control cells by 50% compared to 20-25% growth inhibition of the MCF7/C111S cells (Fig. 6A, Left panel in manuscript in appendix). Dose-response analysis showed that MCF7/C111S cells were resistant to a broad range (0.01-10 nM) of ICI 182,780 concentrations (Fig. 6A, Right panel in manuscript in appendix). On the other hand, estradiol-induced proliferation of MCF7/C11S and control cells was similar (2-fold increase in cell number over a 6-day treatment period; data not shown). The effect of 4-OHT on MCF7/C111S and MCF7/Vec cell proliferation was examined in a time- and dose-response analysis. The response of the cell lines to 4-OHT was similar (Fig. 6B in manuscript in appendix), suggesting that Ubc12C111S expression did not confer cells resistance to growth inhibitory effect of antiestrogens in general. These results suggest that the expression of Ubc12C111S conferred resistance of MCF7 cells to the growth inhibitory effects of ICI 182,780, but disrupting the NEDD8 pathway had no effect on the mitogenic response of MCF7 breast cancer cells to estradiol or the growth inhibitory effects of 4-OHT.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that Uba3 enhances proteasomal degradation of ERα
- Showed that the Neddylation pathway is required for ligand-mediated degradation of ERα
- Discovered that the NEDD8 pathway is required for efficient ubiquitination of ERα
- Generated a human MCF7 breast cancer cell line stably expressing dominant negative Ubc12C111S.
- Determined ERα protein levels in the MCF7 Ubc12C111Sbreast cancer cell line.
- Showed that Ubc12C111S inhibits ICI 182,780-induced down-regulation of ERα
- Established that disrupting the NEDD8 pathway confers antiestrogen resistance in breast cancer cells
- Provided evidence that allowed us to speculate that disruptions in the NEDD8 pathway may provide a mechanism by which breast cancer cells acquire ICI 182,780 resistance while retaining expression of ERα.

REPORTABLE OUTCOMES (List reportable outcomes that have resulted from this research)

Manuscripts

*Fan M, Bigsby RM, Nephew KP 2003 The NEDD8 pathway is required for proteasome mediated degradation of human estrogen receptor-α and essential for the antiproliferation activity of ICI 182,780 in ER-positive breast cancer cells Mol Endocrinol 17:356-365

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CONCLUSIONS

The antiestrogen ICI 182,780 is a drug is used as a second-line endocrine agent in patients who have developed tamoxifen-resistant breast cancer (23). Despite its potent antitumor effects, the drug does not circumvent the development of antiestrogen resistance (24-27). Moreover, the fact that most tumors acquiring ICI 182,780 resistance do so while retaining expression of ERα and estrogen responsiveness (24, 28), suggests that administration of the antiestrogen may possibly lead to the selection of tumor cells defective in ERα down-regulation pathway(s), which in turn may confer a proliferative advantage in either the presence or absence of estrogens. In this context, mechanism underlying persistent expression of ERα in tumors with acquired resistance, such as disruptions in the NEDD8 or other ubiquitin or ubiquitin-like pathways, may thus present an important therapeutic target for future drug intervention.

For the "so what section" (evaluates the knowledge as a scientific or medical product to also be included in the conclusion of this report), the loss of NEDD8 expression during malignant transformation of prostate cancer was recently reported (29). Because our results show an intact NEDD8 pathway is essential for ERα ubiquitination and degradation, we speculate that disruptions in the NEDD8 pathway may provide a mechanism by which breast cancer cells acquire ICI 182,780 resistance while retaining expression of ERα.

In summary, Task 1 is in progress, Task 2 has been completed and Task 3 is in progress.

List of personnel receiving pay from the research effort: Kenneth P. Nephew, Ph.D., Principal Investigator; Meiyun Fan, Ph.D., Postdoctoral Fellow; Teresa Craft, M.S., Research Associate

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APPENDICES

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The NEDD8 Pathway Is Required for Proteasome-Mediated Degradation of Human Estrogen Receptor (ER)-α and Essential for the Antiproliferative Activity of ICI 182,780 in ERα-Positive Breast Cancer Cells

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Steroid hormone receptors, including estrogen receptor-α (ERα), are ligand-activated transcription factors, and hormone binding leads to depletion of receptor levels via preteasome-mediated degradation. NEDD8 (neural precursor cell-expressed developmentally down-regulated) is an ubiquitin-like protein essential for protein processing and cell cycle progression. We recently demonstrated that ubiquitin-activating enzyme (Uba)3, the catalytic subunit of the NEDD8-activating enzyme, inhibits ERα transcriptional activity. Here we report that Uba3-mediated inhibition of ERα transactivation function is due to increased receptor protein turnover. Coexpression of Uba3 with ERα increased receptor degradation by the 26S proteasome. Inhibition of NEDD8 activation and conjugation diminished polyubiquitination of ERα and blocked proteasome-mediated degradation of receptor protein. The antiestrogen ICI 182,780 is known to induce ER degradation. In human MCF7 breast cancer cells modified to contain a disrupted NEDD8 pathway, ICI 182,780 degradation of ERα was impaired, and the antiestrogen was ineffective at inhibiting cell proliferation. This study provides the first evidence linking nuclear receptor degradation with the NEDD8 pathway and the ubiquitin-proteasome system, suggesting that the two pathways can act together to modulate ERα turnover and cellular responses to estrogens. Based on our observation that an intact NEDD8 pathway is essential for the antiproliferation activity of the ICI 182,780 in ERα positive breast cancer cells, we propose that disruptions in the NEDD8 pathway provide a mechanism by which breast cancer cells acquire antiestrogen resistance while retaining expression of ERα. (*Molecular Endocrinology* 17: 356–365, 2003)

ESTROGEN REGULATES DIVERSE biological processes through estrogen receptors (ERα and ERβ) (1). Receptor levels and dynamics have a profound influence on target tissue responsiveness and sensitivity to estrogen (2). ERα is a short-lived protein with a half-life of about 4 h, which is reduced to 3 h by 17β-estradiol (estradiol), and to less than 1 h by the steroidal antiestrogens, ICI 182,780 and ICI 164,384 (3, 4). Receptor turnover rates provide estrogen target cells with the capacity for rapid regulation of receptor levels and thus dynamic hormone responses. An attenuated transcriptional response has been associated with down-regulation of ERα, and receptor up-regulation has been shown to enhance the cellular response to estrogen (2). Nonetheless, mechanisms governing ERα protein levels remain poorly understood.

It has recently been shown that degradation of ERα and other members of the nuclear receptor superfamily occurs through the ubiquitin-proteasome pathway (5). Ubiquitination is a multistep process involving the action of a ubiquitin-activating enzyme (E1 or Uba), a ubiquitin conjugation enzyme (E2 or Ubc), and a ubiquitin ligase (E3) (6). Because the high specificity for target proteins is primarily conferred by E3, regulation of E3 activity may play a crucial role in governing protein degradation *in vivo*. A large number of E3s are cullin-based ubiquitin ligases (7), including SCF (Skp1/Cul1/F-box/ROC1) and VCB (von Hippel-Lindau-Cul2/elongin B/elongin C) complexes. One important level of regulation of these cullin-based ubiquitin ligases involves modification of the cullin subunit with NEDD8, an ubiquitin-like protein (7).

NEDD8 conjugation (neddylation) resembles ubiquitination and involves the action of amyloid precursor protein-binding protein (APP-BP1)/Uba3, a heterodimeric E1-like enzyme, and Ubc12, an E2-like enzyme (8).

Abbreviations: APP-BP1, Amyloid precursor protein-binding protein; AR, androgen receptor; csFBS, charcoal-stripped FBS; E2, ubiquitin conjugation enzyme; E3, ubiquitin ligase; ER, estrogen receptor; estradiol, 17β-estradiol; FBS, fetal bovine serum; HA, hemagglutinin; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEDD8, neural precursor cell-expressed developmentally down-regulated; 4-OHT, 4-hydroxytamoxifen; PR, progesterone receptor; Uba, ubiquitin-activating enzyme; Ubc, ubiquitin-conjugation enzyme.

Whether a ligase is required for neddylation is unknown. To date, the only known substrates of NEDD8 are cullin family members (9, 10). Cullin neddylation is conserved and plays an important regulatory role for cullin-based E3 activity in yeast, plant, and mammalian cells (7, 11–13). Interrupting NEDD8 modification of cullins in mammalian cells has been shown to block ubiquitination of certain proteins involved in different cellular functions, including p27, IκBα, HIFα, and NFκB precursor p105 (14–19). Recent studies have revealed that cullin neddylation is a tightly controlled dynamic process (20–24), and the effect of neddylation on protein polyubiquitination appears to be specific (17, 18).

We recently identified the NEDD8 activating enzyme, Uba3 as an ER-interacting protein and inhibitor of transactivation by steroid nuclear receptors (25). We further demonstrated that an intact neddylation pathway is required for Uba3-mediated inhibition of ER transcriptional activity (25). Taken together with recent reports linking the ubiquitin and NEDD8 pathways (7), our findings raise the intriguing possibility for a role of neddylation in ERα ubiquitination and degradation. Here we show that Uba3 enhances ERα degradation by the 26S proteasome, and expression of dominant-negative mutants of Uba3 or Ubc12 impaired ERα ubiquitination and ligand-induced ERα degradation. Blocking the neddylation pathway with the dominant-negative Ubc in ERα-positive human breast cancer cells inhibited both receptor degradation and the growth inhibitory effect of the antiestrogen ICI 182,780 (known clinically as Faslodex or Fulvestrant). Collectively, these data show that the NEDD8 pathway plays an essential role in ubiquitination and proteasomal degradation of ERα and indicate that disruptions in the pathway may contribute to the development of antiestrogen resistance in human breast cancer.

RESULTS

Uba3 Enhances Proteasomal Degradation of ERα

To test the hypothesis that the neddylation pathway restricts ERα activity by modulating receptor degradation, we transfected HeLa cells with ERα, alone or in combination with an expression vector for Uba3, APP-BP1, or Ubc12, or with an empty vector (pcDNA3.1, Invitrogen, Carlsbad, CA); a green fluorescence protein (GFP) expression vector was cotransfected to serve as a means of normalizing transfection efficiency and sample preparations. Steady-state levels of ERα protein were determined by Western blot analysis. Coexpression of Uba3 decreased ERα protein level but had no effect on GFP expression (Fig. 1A). Treatment of the transfected HeLa cells with MG132, a specific proteasome inhibitor, blocked Uba3-stimulated down-regulation of ERα (Fig. 1B), confirming that the Uba3-induced ERα degradation is through the 26S proteasome. Overexpression of APP-BP1 or Ubc12 had no significant effect on ERα protein levels (data not shown), a result consistent with our previous observation that Uba3 is the limiting factor in neddylation-associated inhibition of ERα transcriptional activity (25).

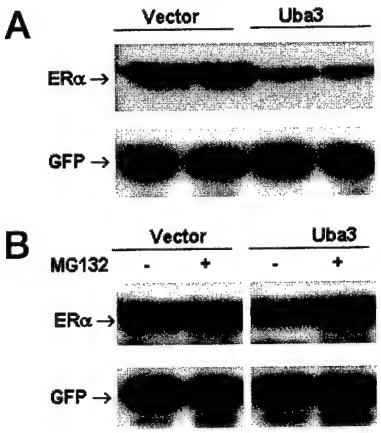

Fig. 1. Uba3 Enhances Proteasomal Degradation of ERα

A, Coexpression of Uba3 decreases ERα protein level in transfected HeLa cells. HeLa cells were transfected with pSG5-ER and pcDNA-Uba3 or pcDNA vector. Whole cell extracts were prepared 24 h post transfection and analyzed by Western blotting to determine ERα protein level. B, Proteasome inhibitor MG132 restores expression level of ERα in cells transfected with Uba3. Transfected HeLa cells (same as in A) were treated with 20 μM MG132 for 6 h before protein extracts and ERα level analysis. GFP was used as an internal control to correct for transfection efficiency and SDS-PAGE loading. Representative results of three independent experiments are shown.

The Neddylation Pathway Is Required for Ligand-Mediated Degradation of ERα

Estradiol stimulates ERα degradation through the ubiquitin-proteasome pathway (26–30). Having established a role for Uba3 in this process, it was important to assess whether neddylation pathway is required for ligand-induced degradation of ERα. To address this issue, we used a dominant-negative mutant of Ubc12 (Ubc12C111S). Due to a single Cys-to-Ser substitution at the active Cys residue, Ubc12C111S forms a stable complex with NEDD8, resulting in sequestration of NEDD8 and inhibition of subsequent NEDD8 conjugation (31, 32). Dominant-negative inhibition of NEDD8 conjugation by Ubc12C111S has been shown to impair efficient ubiquitination and protein degradation (14, 15, 17, 18). Treatment of ERα-transfected HeLa cells with estradiol resulted in a time-dependent decrease in ERα protein levels; receptor levels were reduced by 80% at 6–8 h. (Fig. 2A). In contrast, the effects of estradiol on receptor levels were less dramatic in cells expressing Ubc12C111S, producing a reduction of only 40% by 6–8 h (Fig. 2A). Consistent with this observation, Uba3C216S, a dominant-negative mutant of Uba3 (31, 32), also inhibited estradiol-induced ERα down-regulation (Fig. 2B). Addition of the proteasome inhibitor MG132 before estradiol treatment completely abolished ligand-induced down-regulation of ERα (Fig. 2B), con-

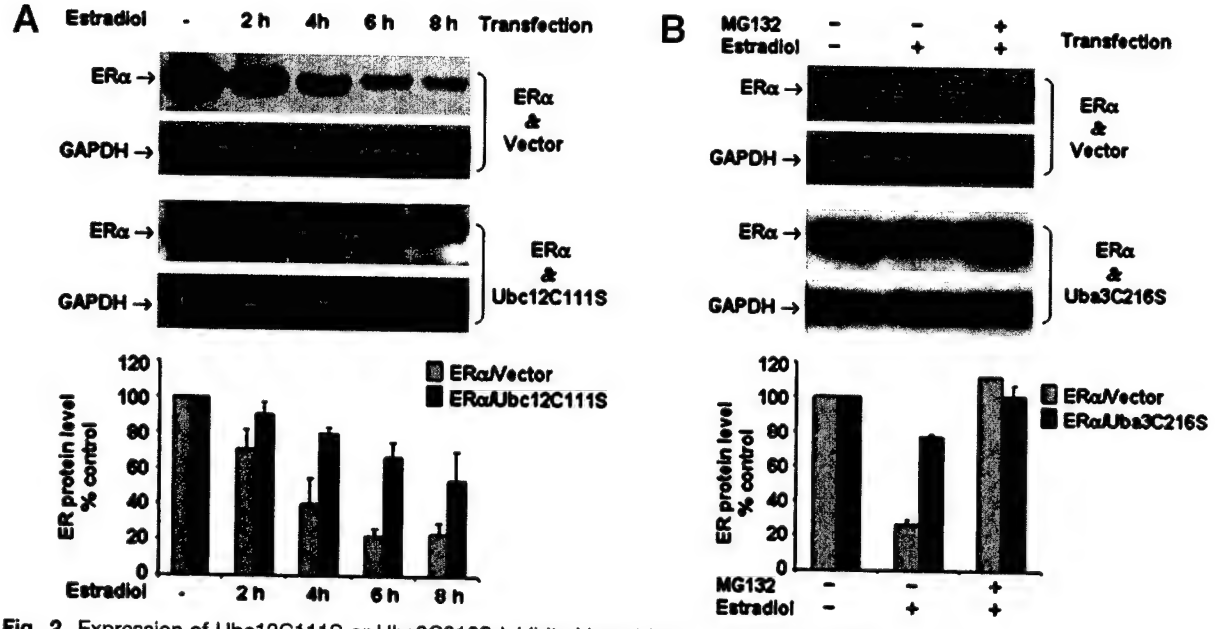

Fig. 2. Expression of Ubc12C111S or Uba3C216S Inhibits Ligand-Induced ERα Degradation

A, HeLa cells were transfected with pSG5-ER and pcDNA vector (*upper panel*) or pcDNA-Ubc12C111S (*lower panel*). Twenty-four hours after transfection, cells were treated with 100 nM estradiol for the indicated times and analyzed for ERα protein level using Western blotting. Relative ERα levels in cells cotransfected with vector (*gray*) or Ubc12C111S (*black*) from two independent experiments are shown in corresponding histogram. B, HeLa cells were transfected with pSG5-ER and pcDNA vector (*upper panel*) or pcDNA-Uba3C216S (*lower panel*). Twenty-four hours after transfection, cells were treated with vehicle or 20 μM MG132 for 1 h followed by incubation with vehicle or 100 nM estradiol for 6 h, as indicated. ERα protein levels were analyzed by immunoblotting. Relative ERα levels in cells cotransfected with vector (*gray*) or Uba3C216S (*black*) from three independent experiments are shown in corresponding histogram. GAPDH was used as an internal control to correct SDS-PAGE loading.

firming that exogenous ERα in HeLa cells undergoes proteasome-dependent degradation in response to estradiol. Collectively, these results demonstrate that a functional NEDD8 pathway is required for efficient, ligand-induced, proteasome-mediated degradation of ERα.

The NEDD8 Pathway Is Required for Efficient Ubiquitination of ERα

Having established a role for Uba3 and Ubc12 in ERα down-regulation, it was important to examine the effect of NEDD8 on receptor ubiquitination. HeLa cells were cotransfected with ERα and hemagglutinin (HA)-tagged ubiquitin, along with wild-type Ubc12 or Uba3 or the corresponding mutant forms of these neddylation enzymes (Ubc12C111S or Uba3C216S). At 24 h post transfection, cells were treated with MG132 or vehicle, followed by estradiol treatment. Immunoprecipitation assays using an anti-ERα antibody were performed and the levels of ubiquitinated ERα in the precipitated immunocomplex were assessed by Western blotting with an anti-HA antibody. The polyubiquitinated ERα exhibited a ladder of higher molecular weight species on the blot membrane (Fig. 3). Expression of dominant-negative Ubc12C111S or Uba3C216S markedly decreased ERα ubiquitination in either the absence (Fig. 3, *left panel*) or presence of estradiol and MG132 (Fig. 3, *right panel*), compared with cells transfected with control vector or wild-type Ubc12 or Uba3. These results suggest that a functional neddylation pathway is required for the efficient ubiquitination of ERα.

ERα Protein Levels in MCF7 Breast Cancer Cell Lines Stably Expressing Dominant-Negative Ubc12C111S

MCF7 human breast cancer cells express high levels of ERα and proliferate in response to estrogen treatment (33, 34), providing a model to study endogenous ERα function. To further investigate the role of neddylation in ERα function under physiological relevant conditions, we transfected Ubc12C111S into MCF7 cells and established the stable cell line MCF7/C111S. As a control, MCF7/Vec (MCF7 cells stably transfected with empty vector) was also established. Expression of the Ubc12C11S mutant protein in MCF7/C111S cells was confirmed by Western blotting and, consistent with a previous report (31), the mutant was detected as 26- and 31-kDa proteins (Fig. 4, lanes 3–8). In the regular growth medium containing phenol red and 10% fetal bovine serum (FBS), the level of ERα in MCF7/Vec cells was very low; after 3 d of culture in hormone-free medium containing 3% dextran-coated charcoal-stripped FBS (cs-FBS) and no phenol red, ERα expression was dramatically increased (Fig. 4, lanes 1 and 2). The culture medium (regular growth medium *vs.* hormone-free me-

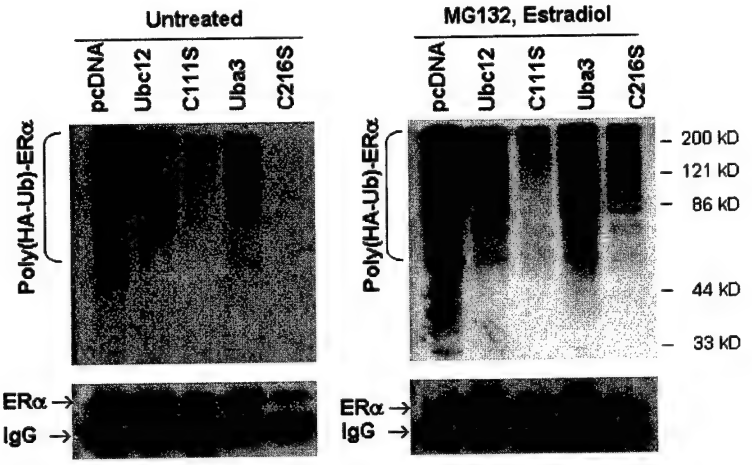

Fig. 3. An Intact NEDD8 Pathway Is Required for Efficient ERα Ubiquitination

HeLa cells were transfected with pSG5-ER, and pcDNA-HA-Ubiquitin, alone with indicated construct. Twenty-four hours after transfection, cells were either untreated (*left panel*) or treated with 20 μM MG132 for 1 h followed by 100 nM estradiol exposure for 3 h (*right panel*). Protein extracts were prepared and subjected to immunoprecipitation using anti-ERα antibody. Polyubiquitinated ERα was detected by Western blotting using anti-HA antibody, and was visualized as a ladder of higher molecular weight species on the blot. The blot was striped and reprobed by anti-ERα antibody to assess the amount of precipitated ERα (*lower panels*). The heavy chain of the anti-ERα IgG used for immunoprecipitation exhibits a 57-kDa band in the ERα blot. Representative results of three independent experiments are shown.

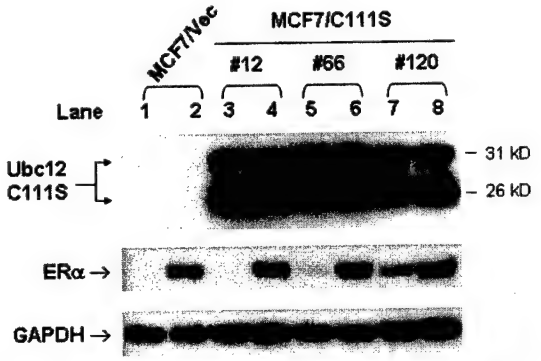

Fig. 4. The Expression of Ubc12C111S and ERα in Three Independent MCF7/C111S Clones

MCF7/C111S cells stably expressing mutant Ubc12C111S were maintained in growth medium (lanes 1, 3, 5, and 7) or hormone-free medium for 3 d (lanes 2, 4, 6, and 8) and analyzed by immunoblotting using anti-HA (*upper panel*) or anti-ERα (*lower panel*) antibodies, respectively. GAPDH was used as an internal control to correct for SDS-PAGE loading.

dium) showed no effect on the expression level of Ubc12C111S. In three MCF7/C111S clones, receptor levels varied among the clones and, when cultured in growth medium, detectable ERα was seen in two of the three clones (Fig. 4, lanes 5 and 7). When cultured in estrogen-free medium, however, ERα levels were high in all three clones (Fig. 4, lanes 4, 6, 8).

Ubc12C111S Inhibits ICI 182,780-Induced Down-Regulation of ERα

In contrast to estradiol, which down-regulates ERα in target tissues through both transcriptional and posttranslational mechanism (35, 36), the pure antiestrogen ICI 182,780 causes ERα protein degradation without affecting ERα mRNA levels (3, 36). Based on our observations that the NEDD8 pathway is essential for ERα degradation in transfected HeLa cells (Fig. 2), it was of interest to examine the effect of the antiestrogen on ERα degradation in MCF7/C111S cells. Cells were cultured in hormone-free medium for 3 d before ICI 182,780 treatment. Under this condition, comparable amounts of ERα were observed in MCF7/C111S and MCF7/Vec cells (compare 0-h lanes in Fig. 5A). Treatment with ICI 182,780 rapidly (by 1 h) decreased ERα levels in the MCF7/Vec cells; by 4 h post treatment, the levels of ERα were reduced by 95% (Fig. 5A). In the MCF7/C111S cells, the effects of ICI 182,780 on ERα levels were much less dramatic (Fig. 5A). Thus, although ER degradation was not completely inhibited by expression of the dominant-negative Ubc12C111S, these results confirm our observations using transient transfection in HeLa cells and further suggest that the NEDD8 pathway is required for efficient degradation of endogenous ERα. To examine the effect of another antiestrogen on ERα degradation in this system, cells were cultured in the presence of various doses of 4-hydroxytamoxifen (4-OHT) and ERα levels were examined. In both MCF7/Vec and MCF7/C111S cells, ERα levels remained unchanged or were slightly increased after treatment with 4-OHT (Fig. 5B). Stabilization of ERα by tamoxifen has been reported by others (30), perhaps due to inhibition of the basal rate of ER degradation by the antiestrogen.

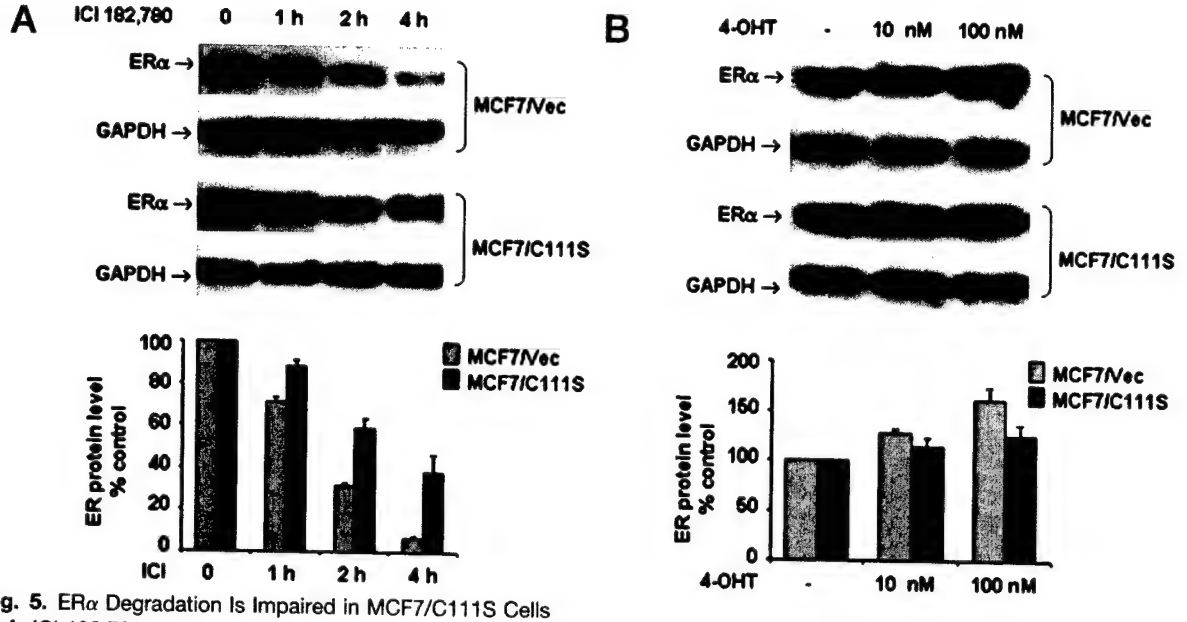

Fig. 5. ERα Degradation Is Impaired in MCF7/C111S Cells

A, ICI 182,780-induced ERα degradation is impaired in MCF7/C111S cells. MCF7/Vec (*upper panel*) and MCF7/C111S cells (*lower panel*) were cultured in hormone-free medium for 3 d and treated with 1 nM ICI 182,780 for the indicated times. B, 4-OHT does not cause ERα degradation in MCF7 cells. MCF7/Vec (*upper panel*) and MCF7/C111S cells (*lower panel*) were cultured in hormone-free medium for 3 d and treated with indicated doses of 4-OHT for 6 h. ERα protein levels were determined by Western blotting with anti-ERα antibody. The histogram shows the relative ERα levels after ICI 182,780 or 4-OHT treatment. Relative ERα levels in MCF7/vec (*gray*) from three independent experiments or MCF7/C111S (*black*) from three independent MCF7/C111S clones are shown in corresponding histogram. GAPDH was used as an internal control to correct SDS-PAGE loading.

Disrupting the NEDD8 Pathway Confers Antiestrogen Resistance in Breast Cancer Cells

Estradiol is mitogenic in MCF7 cells and stimulates cell proliferation through activation of ERα (37). The pure antiestrogen ICI 182,780, on the other hand, blocks ERα-mediated transactivation and induces ERα protein degradation, resulting in growth inhibition of breast cancer cells (38). Because expression of Ubc12C111S inhibited ICI 182,780-induced ERα down-regulation (Fig. 5A), we examined the growth inhibitory effect of ICI 182,780 in MCF7/C111S cells. No significant difference was observed in basal cell proliferation rates between MCF7/C111S and MCF7/Vec cells in hormone-free medium (data not shown). Treatment with the antiestrogen (1 nM) inhibited the basal cell growth of MCF7 and MCF7/Vec cells (Fig. 6A). In contrast, MCF7/C111S cells were partially resistant to ICI 182,780. Specifically, over an 8-d period, the antiestrogen inhibited the growth of control cells by 50% compared with 20–25% growth inhibition of the MCF7/C111S cells (Fig. 6A, *left panel*). Dose-response analysis showed that MCF7/C111S cells were resistant to a broad range (0.01–10 nM) of ICI 182,780 concentrations (Fig. 6A, *right panel*). On the other hand, estradiol-induced proliferation of MCF7/C11S and control cells was similar (2-fold increase in cell number over a 6-d treatment period; data not shown). The effect of 4-OHT on MCF7/C111S and MCF7/Vec cell proliferation was examined in a time- and dose-response analysis. The response of the cell lines to 4-OHT was similar (Fig. 6B), suggesting that Ubc12C111S expression did not confer cells resistance to growth inhibitory effect of antiestrogens in general. These results suggest that the expression of Ubc12C111S conferred resistance of MCF7 cells to the growth inhibitory effects of ICI 182,780, but disrupting the NEDD8 pathway had no effect on the mitogenic response of MCF7 breast cancer cells to estradiol or the growth inhibitory effects of 4-OHT.

DISCUSSION

ERα is a short-lived protein whose degradation is primarily mediated by the ubiquitin-proteasome pathway (26–30). The recently described ubiquitin-like pathways, including the NEDD8 and SUMO (small ubiquitin-like modifier) conjugation systems (39), have been implicated in nuclear receptor regulation (40–44) and the NEDD8 pathway has been shown to enhance protein polyubiquitination (12, 14–19, 45–47). Our previous investigation into the role of the NEDD8 pathway in nuclear hormone receptor regulation showed that Uba3, the catalytic subunit of the NEDD8 activating enzyme complex, interacts with ERα and inhibits receptor function (25). Here we report that Uba3-mediated inhibition of ERα transactivation is due to increased receptor turnover and that an intact neddylation pathway is essential for ERα ubiquitination and degradation. By impairing the NEDD8 path-

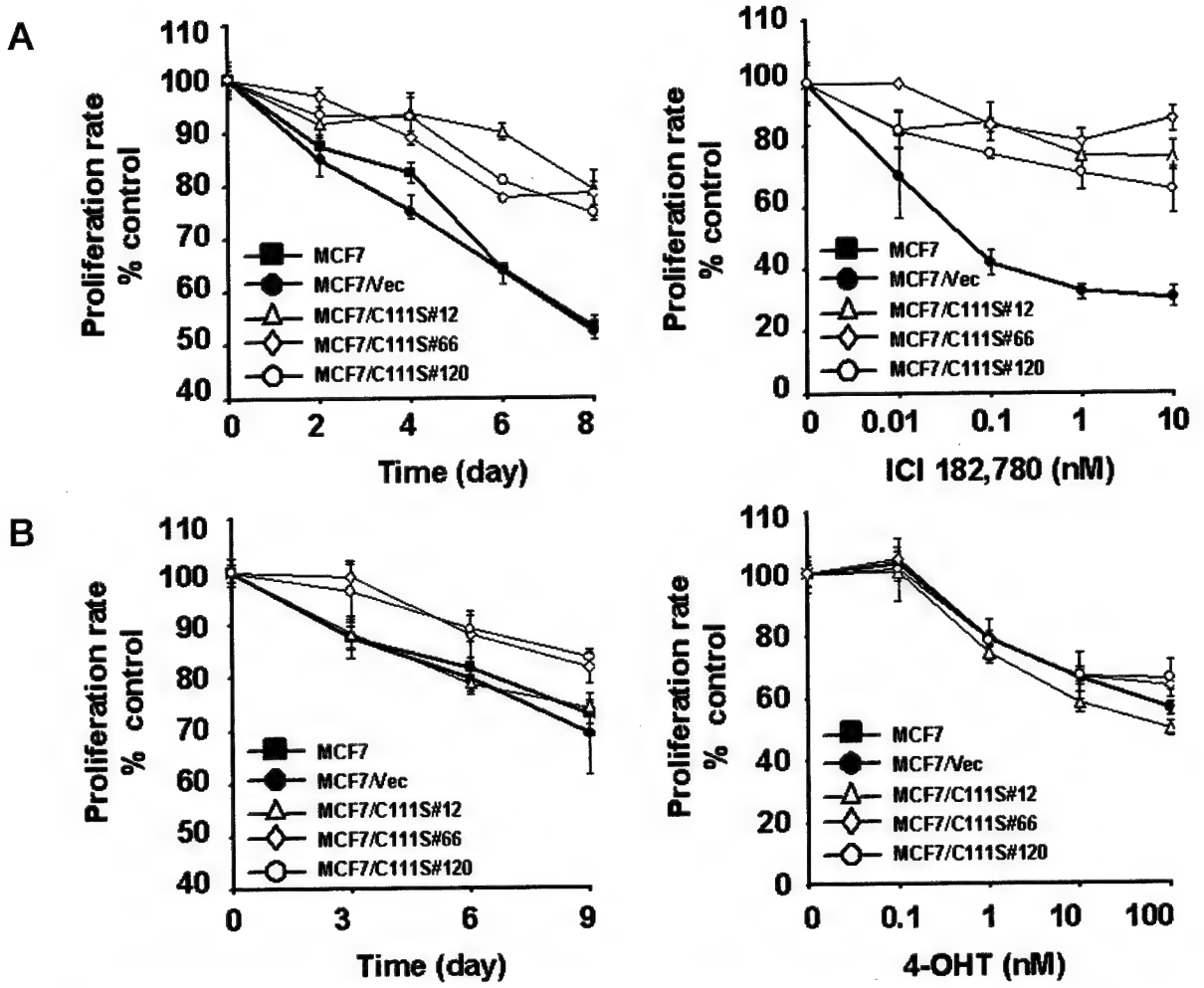

Fig. 6. Interruption of the NEDD8 Pathway Confers Resistance to ICI 182,780 in Human Breast Cancer Cells

A, Time- and dose-dependent growth inhibition of ICI 182,780. For time-response analysis, cells were treated with 1 nM ICI 182,780 and cell numbers were determined 0, 2, 4, 6, and 8 d after drug exposure. For dose-response assay, cells were treated with indicated doses of ICI 182,780 and cell numbers were determined on d 7. B, Time- and dose-dependent antiproliferative effect of 4-OHT. For time-response analysis, cells were treated with 10 nM 4-OHT and cell numbers were determined 0, 3, 6, and 9 d later. For the dose-response assay, cells were treated with indicated doses of 4-OHT and cell numbers were determined on d 7. For all assays, cells were cultured in hormone-free medium for 3 d before treatment and cell numbers were determined by MTT assay. Relative proliferation rate was expressed as percentage of cells grown in hormone-free medium. Each experiment was repeated three times in quadruplicate.

way in human MCF7 breast cancer cells, we demonstrated that the cells became resistant to the growth inhibitory effects of ICI 182,780. Thus, our data suggest that neddylation plays an important role in ERα degradation and we speculate that alterations in the NEDD8 pathway may provide a mechanism by which tumors can acquire antiestrogen resistance.

Several recent studies have focused on the role of the ubiquitin-proteasome pathway in nuclear receptor down-regulation (26–30). Enhancement of ERα ubiquitination by estradiol was first reported by Nirmala and Thampan (48), and Nawaz *et al.* (27) showed that a functional ubiquitin-proteasome system is required for ERα degradation. Both basal and ligand-induced ERα ubiquitination occurs at the nuclear matrix (49), but how ERα is targeted for ubiquitination has not been fully established. Previously, we had shown that Uba3 interacts directly with ER and that this interaction is augmented by estradiol (25). Here, we show that overexpression of Uba3 enhanced degradation of ERα and that disruption of Uba3 activity reduces estradiol-induced receptor degradation. Taken together, these data support a role for Uba3 in the regulation of basal as well as ligand-induced ERα turnover.

The present study is the first to link the NEDD8 pathway to ubiquitination of ERα. The exact mechanism connecting the two pathways, however, remains unclear. The only known substrates for direct neddylation are members of the cullin family (10). Some of the cullins have been identified as core subunits of specific ubiquitin ligase complexes (7). Mechanistically, conjugation of NEDD8 to cullins may up-regulate

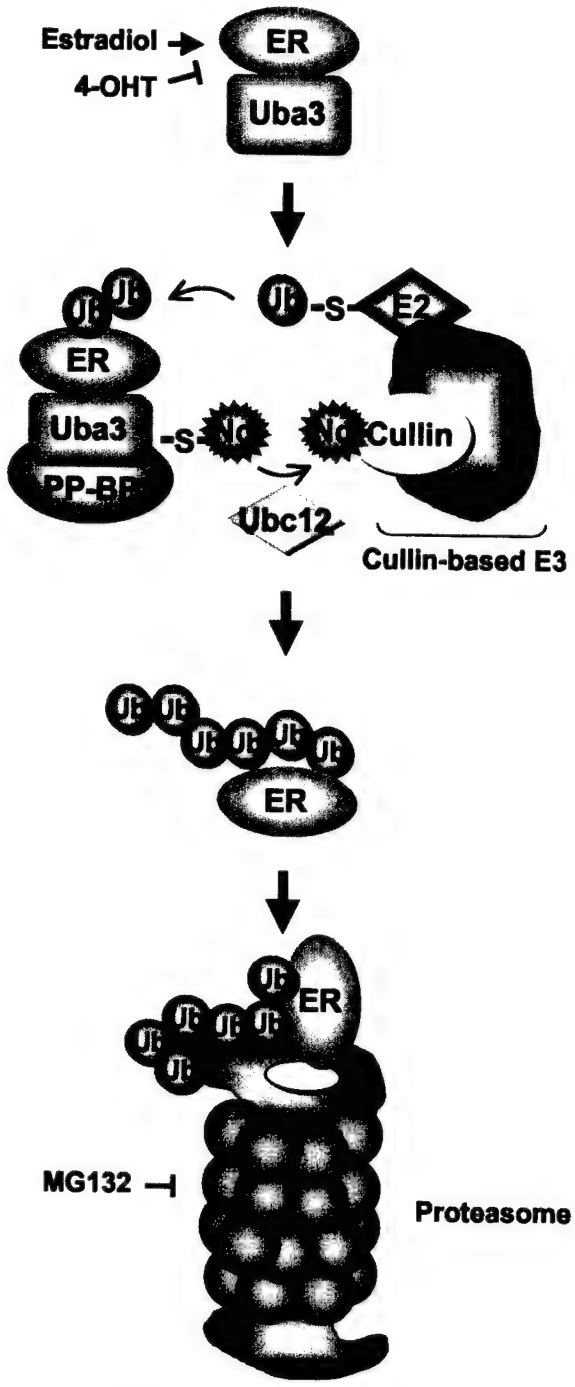

Fig. 7. Hypothetical Model Depicting the Role of Neddylation Pathway in Proteasome-Mediated Degradation of ERα

The physical interaction between Uba3 and ERα promotes the functional recruitment and activation of a cullin-based ubiquitin-protein ligase to augment receptor polyubiquitination. Uba3 and APP-BP1, the heterodimeric activating enzyme for NEDD8, and Ubc12, the NEDD8 conjugating enzyme, promote cullin NEDD8 modification of specific ubiquitin E3 ligases. Neddylated cullins enhance the formation and activity of the ubiquitin E2-E3 complex. The potency of ERα-Uba3 interaction appears to correlate with ERα turnover rate. In the absence of ligand, ERα interacts weakly with Uba3, resulting in basal ubiquitination and degradation of ERα; however, estradiol augments the ERα-Uba3 interaction to enhance ERα ubiquitination. On the other hand, 4-OHT interrupts the ERα-Uba3 interaction and stabilizes ERα, and MG132 blocks ERα degradation by inhibiting proteasome activity. APP-BP1, Amyloid precursor protein-binding protein; E2, ubiquitin conjugation enzyme; E3, ubiquitin protein ligase; estradiol, 17β-estradiol; Nd, neural precursor cell-expressed developmentally down-regulated (NEDD8); ↓ and ⊥, Stimulation and inhibition, respectively.

ubiquitin ligase activity of specific E3s by facilitating the formation of an ubiquitin E2-E3 complex (45). In this regard, the interaction between Uba3 and ERα could result in the functional recruitment and activation of a cullin-based ubiquitin-protein ligase, which, in turn, targets ERα for degradation by the ubiquitin-proteasome system. The hypothetical model depicting the role of neddylation pathway in proteasome-mediated degradation of ERα is shown in Fig. 7. Together with our previously reported data (25), these observations indicate that such targeted degradation of ERα leads to reduced hormonal responsiveness.

In addition to its effect on ERα, Uba3 inhibits the transactivation function of other steroid receptors, ERβ, androgen receptor (AR) and progesterone receptor (PR) (25). Others have reported that NEDD8 interacts with aryl hydrocarbon receptor and the interaction affects the transcriptional activity and stability of the receptor protein (40). Furthermore, the NEDD8 protein has been found to colocalize with AR (50). Together with the observations that turnover of ER, AR, PR, and aryl hydrocarbon receptor occurs via degradation by the 26S proteasome (28, 51–53), these results provide compelling evidence for integration of the neddylation and ubiquitin-proteasome pathways in steroid hormone action. Because receptor levels can have a profound influence on target tissue responsiveness to hormone, NEDD8 and ubiquitin pathways, by modulating receptor protein turnover, could play important roles in determining and perhaps limiting cellular responses to steroid hormones and antihormones.

The antiestrogen ICI 182,780 is a 7α-alkylsulfinyl analog of estradiol lacking agonist activity (54). The drug is used as a second-line endocrine agent in patients who have developed tamoxifen-resistant breast cancer (38). Although the drug clearly displays complex pharmacology, rapid degradation of ERα protein has been associated with the antiproliferative effects of ICI 182,780 on breast cancer cells (38, 54). Despite its potent antitumor effects, the drug does not circumvent the development of antiestrogen resistance (55–58). Moreover, the fact that most tumors acquiring ICI 182,780 resistance do so while retaining expression of ERα and estrogen responsiveness (55, 59) suggests that administration of the antiestrogen may possibly lead to the selection of tumor cells defective in ERα down-regulation pathway(s), which in turn may confer a proliferative advantage in either the presence or absence of estrogens. Mechanism underlying persistent expression of ERα in tumors with acquired resis-

tance may thus present an important therapeutic target for future drug intervention. In this context, the loss of NEDD8 expression during malignant transformation of prostate cancer was recently reported (60). Because our results show an intact NEDD8 pathway is essential for ERα ubiquitination and degradation, we speculate that disruptions in the NEDD8 pathway may provide a mechanism by which breast cancer cells acquire ICI 182,780 resistance while retaining expression of ERα.

MATERIALS AND METHODS

Materials

The following antibodies and reagents were used in this study: anti-ER (HC20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-HA (3F10; Roche Molecular Biochemicals, Indianapolis, IN); anti-GFP (GFP01, NeoMarkers, Inc., Fremont, CA); anti-GAPDH (glyceraldehyde phosphate dehydrogenase; Chemicon International, Inc., Temecula, CA); anti-rabbit IgG and protein G-agarose beads (Oncogene Research Products, San Diego, CA); SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL); protease inhibitor cocktail set III (Calbiochem-Novabiochem Corp., San Diego, CA); Bio-Rad Laboratories, Inc. (Hercules, CA) protein assay kit; FBS and csFBS (HyClone Laboratories, Inc., Logan, UT); LipofectAMINE Plus Reagent, geneticin, and other cell culture reagents were from Life Technologies, Inc. (Rockville, MD). Estradiol, 4-OHT, MG132, and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO). ICI 182,780 was purchased from Tocris Cookson Ltd. (Ellisville, MO).

Plasmid Construction

The construction of pSG5-ER(HEGO), pcDNA-Uba3, pcDNA-HA-Uba3C216S, pcDNA-HA-Ubc12, and pcDNA-HA-Ubc12C111S was described previously (25). The pcDNA-HA-ubiquitin was kindly provided by Y. Xiong (61). The pCMV (cytomegalovirus)-GFP was purchased (Promega Corp., Madison, WI).

Cell Lines

The human cervical carcinoma cell line, HeLa, and the breast cancer cell line, MCF-7 were purchased from ATCC (Manassas, VA). HeLa cells were maintained in MEM with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% FBS. MCF7 cells were maintained in MEM with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, 6 ng/ml insulin, and 10% FBS. Before experiments involving in transient transfection and hormone treatment, cells were cultured in hormone-free medium (phenol red-free MEM with 3% csFBS) for 3 d.

Transient Transfection Assays

HeLa cells were cultured in hormone-free medium for 3 d and transfected with equal amount of total plasmid DNA (adjusted by corresponding empty vectors) by using LipofectAMINE Plus Reagent according to the manufacturer's guidelines. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were cultured in hormone-free medium. All cells were also cotransfected with pCMV-GFP as internal control to correct for transfection efficiency and SDS-PAGE loading.

Stable Transfection

MCF7 cells were transfected with pcDNA-HA-Ubc12C111S or empty vector by using LipofectAMINE Plus Reagent and selected in growth medium containing 0.5 mg/ml geneticin for 3 wk. Drug-resistant colonies were chosen and expanded in growth medium containing 0.3 mg/ml geneticin. The expression of HA-Ubc12C111S in the stable cell lines (MCF7/C111S) was detected by Western blotting with anti-HA antibody. Geneticin-resistant clones from vector transfectants (MCF7/Vec) were pooled, maintained in growth medium containing 0.3 mg/ml geneticin, and used as control cells.

Preparation of Cell Extracts and Immunoblotting

Whole cell extracts were prepared by suspending cells (~2×10^6) in 0.1 ml of ice-cold lysis buffer (25 mM HEPES, pH 7.5; 0.3 M NaCl; 0.2% sodium dodecyl sulfate; 0.5% sodium deoxycholate; 0.2 mM EDTA; 0.5 mM dithiothreitol; 0.1% Triton X-100; 10 μl protease inhibitor cocktail set III). After 15 min on ice, extracts were sonicated (3 × 10 sec), insoluble material was removed by centrifugation (15 min at 12,000 × *g*), and protein concentration in the supernatant was determined using the Bio-Rad Laboratories, Inc. protein assay kit. The protein extracts were mixed with 1/4 vol of 5× electrophoresis sample buffer and boiled for 5 min at 90 C. Protein extract (50 μg per lane) was then fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies. Primary antibody was detected by horseradish peroxidase-conjugated second antibody and visualized using enhanced SuperSignal West Pico Chemiluminescent Substrate. The band density of exposed films was evaluated with ImageJ software (http://rsb.info.nih.gov/ij/).

Immunoprecipitation

For immunoprecipitation, 500 μg whole cell extract was diluted to protein concentration of 1 μg/μl using PBS containing protease inhibitor cocktail and incubated with 5 μl anti-rabbit IgG and 20 μl protein G-agarose beads for 1 h at 4 C. After centrifugation at 12,000 × *g* for 15 sec, the precleared supernatants were incubated with 5 μl anti-ER antibody overnight at 4 C, followed by another 1-h incubation with 30 μl protein G-agarose beads. The beads were then pelleted by brief centrifugation, washed three times with PBS and once with PBS containing 0.4 M NaCl, and resuspended in 30 μl SDS-PAGE loading buffer for SDS-PAGE and Western blotting.

Cell Proliferation Assays

To assess the effects of estradiol, ICI 182,780, or 4-OHT on cell proliferation, cells (1000/well) were plated in 96-well dishes in hormone-free medium for 3 d before drug exposure. For time-response analysis, cell numbers were determined by MTT assay (62) at indicated times after drug treatment; and for dose-response analysis, cell number was determined by MTT assay at d 7.

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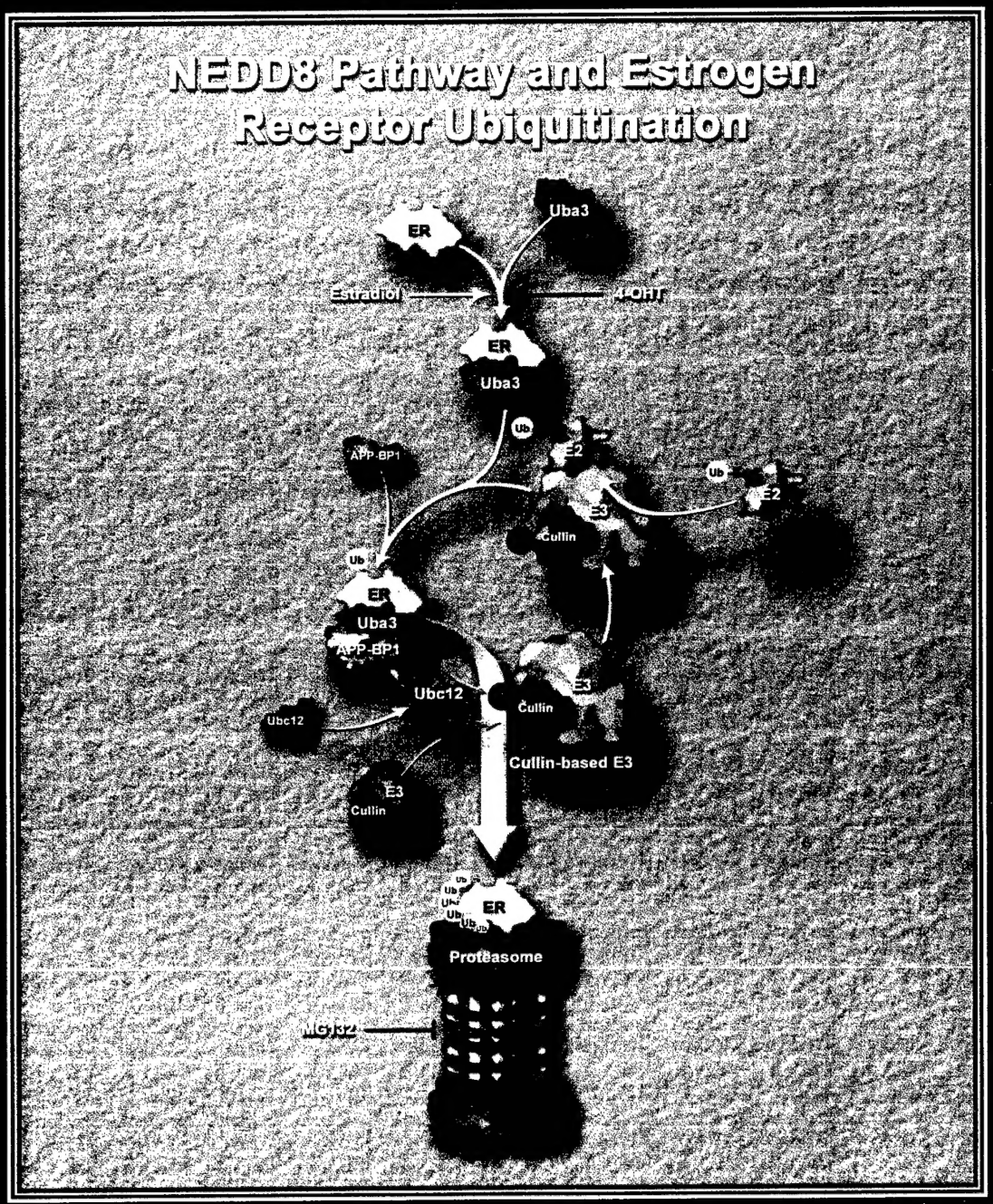

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216 **The Activating Enzyme of NEDD8 Inhibits Steroid Receptor Function**

Meiyun Fan[1], Xinghua Long[1,2], Jason A. Bailey[1], Chad A. Reed[1], Elizabeth Osborne[1], Edward A. Gize[1], Eric A. Kirk[1], Robert M. Bigsby[2,3] and Kenneth P. Nephew[1,2,3]
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Coregulator proteins, coactivators and corepressors, have a profound influence on steroid receptor activity and play a role in regulating receptor levels. To identify novel coregulators of nuclear receptors, we used the ligand binding and hinge region of ERα as bait in a yeast two-hybrid screen of a cDNA library derived from rat uterine luminal epithelium. We report the cloning and characterization of a cDNA encoding a protein homologous to yeast and human Uba3, the catalytic subunit of the activating enzyme of the ubiquitin-like NEDD8 conjugation pathway (known as neddylation). Sequence analysis revealed that Uba3 contains multiple nuclear receptor interacting motifs (NR boxes), which are known to mediate interactions between coregulatory proteins and ligand-activated nuclear receptors. Yeast two-hybrid and GST pull-down assays demonstrated that Uba3 directly interacts with ligand-occupied ERα and ERβ. Transient transfection of Uba3 in mammalian cells inhibited ER-mediated transactivation in a time-dependent fashion; Uba3 had no effect on the initial events of transcriptional activation by liganded ER but it blocked the progressive increase in target gene expression during continuous stimulation. Uba3 also inhibited transactivation by AR and PR in mammalian cells, but had no effect on a steroid receptor independent transactivation pathway. An enzymatically silent form of Uba3 did not inhibit ER-induced transcription and a Uba3 binding fragment of APP-BP1, the other subunit of the NEDD8-activating enzyme, partially overcame Uba3 mediated inhibition, demonstrating that the neddylation activity of Uba3 is required for its inhibition of steroid receptor transactivation. Thus, Uba3 inhibits transcription induced by steroid hormone receptors through a novel mechanism that involves the neddylation pathway. Understanding the mechanisms controlling hormone responsiveness of target tissues, such as the uterus and mammary gland, may lead to novel insights of therapeutic intervention.

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OR14-5

Role for the Neddylation Pathway in Estrogen Receptor Ubiquitination and Degradation.

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Estrogen receptors (ERα and ERβ) are ligand activated transcription factors that regulate diverse biological processes. Receptor levels and dynamics have a profound effect on target tissue sensitivity to estrogen, and ligand binding influences ER stability. Receptor degradation occurs through the ubiquitin-proteasome pathway and may play an important role in the duration of ligand-induced responses. NEDD8, a ubiquitin-like molecule that plays an important role in regulating cell cycle progression, targets cullin family proteins, a major component of E3 ubiquitin ligase complexes. NEDD8 is an essential mediator of ubiquitination; apart from cullins, the target(s) of NEDD8 are unknown. Recently, we reported that Uba3, an activating enzyme of NEDD8, downregulated transactivation by nuclear receptors and that the neddylation activity of Uba3 was required for inhibition of ER-mediated transcription (Mol Endo 16(2), 2002). In the present study, we investigated the mechanism underlying Uba3-mediated suppression of ERα. To determine if the receptor is a direct target for neddylation, HeLa cells were cotransfected with ERα and Myc-tagged NEDD8. Coimmnoprecipitation (CoIP) assays were performed using antibodies against ERα or c-Myc. Under these conditions, we were unable to detect any neddylated ERα, suggesting that the receptor is not a direct substrate for NEDD8. To determine if Uba3 acts indirectly by enhancing receptor ubiquitination and degradation, HeLa cells were cotransfected with ERα and HA-tagged ubiquitin and subjected to Western blot and CoIP analysis. In addition, to block the neddylation pathway, HeLa cells were transfected with either Uba3C216S, an inactive mutant of Uba3, APP-BP1(443-534), a fragment of APP-BP1 that dimerizes with Uba3 to form an inactive enzyme complex, or Ubc12C111S, an inactive mutant of the conjugation enzyme of neddylation, Ubc12. Western and CoIP analysis demonstrated that expression of Uba3 induced degradation of the receptor; furthermore, expression of Uba3C216S, APP-BP1(443-534), or Ubc12C111S reduced ubiquitination of ERα. Taken together, our observations suggest that the neddylation pathway restricts ER-mediated signaling by facilitating ubiquitination and degradation of the receptor protein.

Role for the Neddylation Pathway in Estrogen Receptor Ubiquitination and Degradation

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Estrogen receptors (ERα and ERβ) are ligand activated transcription factors that regulate diverse biological processes. Receptor levels and dynamics have a profound effect on target tissue sensitivity to estrogen, and ligand binding influences ER stability. Receptor degradation occurs through the ubiquitin-proteasome pathway and may play an important role in the duration of ligand-induced responses. NEDD8, a ubiquitin-like molecule that plays an important role in regulating cell cycle progression, targets cullin family proteins, a major component of E3 ubiquitin ligase complexes. NEDD8 is an essential mediator of ubiquitination; apart from cullins, the target(s) of NEDD8 are unknown. Recently, we reported that Uba3, an activating enzyme of NEDD8, downregulated transactivation by nuclear receptors and that the neddylation activity of Uba3 was required for inhibition of ER-mediated transcription (Mol Endo 16(2), 2002). In the present study, we investigated the mechanism underlying Uba3-mediated suppression of ERα. To determine if the receptor is a direct target for neddylation, HeLa cells were cotransfected with ERα and Myc-tagged NEDD8. Coimmnoprecipitation (CoIP) assays were performed using antibodies against ERα or c-Myc. Under these conditions, we were unable to detect any neddylated ERα, suggesting that the receptor is not a direct substrate for NEDD8. To determine if Uba3 acts indirectly by enhancing receptor ubiquitination and degradation, HeLa cells were cotransfected with ERα and HA-tagged ubiquitin and subjected to Western blot and CoIP analysis. In addition, to block the neddylation pathway, HeLa cells were transfected with either Uba3C216S, an inactive mutant of Uba3, or Ubc12C111S, an inactive mutant of the conjugation enzyme of neddylation, Ubc12. Western and CoIP analysis demonstrated that expression of Uba3 induced degradation of the receptor; furthermore, expression of Uba3C216S or Ubc12C111S reduced ubiquitination of ERα. Taken together, our observations suggest that the neddylation pathway restricts ER-mediated signaling by facilitating ubiquitination and degradation of the receptor protein.
Keywords: Estrogen receptor (ER); Ubiquitin; Proteolysis